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Comparison of Methods for Detection and Isolation of Cold- and Freeze-Stressed *Escherichia coli* O157:H7 in Raw Ground Beef†

PINA M. FRATAMICO* AND LORI K. BAGI

U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038, USA

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ABSTRACT

A comparison was made of the relative efficiencies of three enrichment media, RapidChek *Escherichia coli* O157:H7 enrichment broth (REB), R&F broth (RFB), and modified *E. coli* broth containing novobiocin (mEC+n), and four selective plating media for detection of cold- and freeze-stressed *E. coli* O157:H7 in raw ground beef. Ground beef (25 g) was inoculated with *E. coli* O157:H7 at ≤0.5 and ≤2 CFU/g, and samples were then enriched immediately or were stored at 4°C for 72 h or at −20°C for 2 weeks and then enriched. After 8 or 20 h of enrichment, the cultures were plated onto R&F *E. coli* O157:H7 chromogenic plating medium, cefixime-tellurite sorbitol MacConkey agar, CHROMagar O157, and Rainbow agar O157 and tested using the RapidChek *E. coli* O157 lateral flow immunoassay and a multiplex PCR assay targeting the *E. coli* O157:H7 *eae*, stx_1, and stx_2 genes. Recovery of *E. coli* O157:H7 on the four agar media was 4.0 to 7.9 log CFU/ml with the REB enrichment, 1.4 to 7.4 log CFU/ml with RFB, 1.7 to 6.7 log CFU/ml with mEC+n incubated at 42°C, and 1.3 to 3.3 log CFU/ml from mEC+n incubated at 35°C. The percentages of positive ground beef samples containing nonstressed, cold-stressed, and freeze-stressed *E. coli* O157:H7 as obtained by plating, the immunoassay, and the PCR assay were 97, 88, and 97%, respectively, with REB, 92, 81, and 78%, respectively, with RFB, 97, 58, and 53%, respectively, with mEC+n incubated at 42°C, and 22, 31, and 25%, respectively, with mEC+n incubated at 35°C. Logistic regression analyses of the data indicated significant main effects of treatment, type of medium, enrichment time, inoculum concentration, and detection method. In particular, a positive result was 1.1 times more likely to occur after 20 h of enrichment than after 8 h, 25 times more likely with RFB and REB than with mEC+n at 35°C, 3.7 times more likely with an initial inoculum of ≤2.0 CFU/g than with ≤0.5 CFU/g, 2.5 to 3 times more likely using freeze-stressed or nonstressed bacteria than with cold-stressed bacteria, and 2.5 times more likely by plating than by the immunoassay or the PCR assay. REB had better overall performance for enrichment of cold- and freeze-stressed *E. coli* O157:H7 present in ground beef than did the other media examined.

Escherichia coli O157:H7 is an important foodborne pathogen associated with cases and outbreaks of bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. Foods of bovine origin, in particular ground beef, are major vehicles of infection, although other foods such as raw vegetables and apple cider also have been linked to outbreaks *(15)*. Sensitive and reliable methods for detection of this organism are needed by the food industry and regulatory agencies to prevent foods contaminated with *E. coli* O157:H7 from reaching the consumer. Methods employed for detection of foodborne pathogens include (i) traditional culture techniques, which rely on selective enrichment of the target organism in liquid medium, plating onto selective or differential agar(s), and confirmation of the isolate using a series of biochemical and other tests, and (ii) rapid techniques that generally still rely on a selective enrichment step that is then followed by detection of microbial antigens using immunological techniques such as lateral flow assays and enzyme linked immunosorbent assays or detection of genetic markers using techniques that include PCR, which amplifes DNA.

Various traditional, genetic, and immunological methods or combinations of these types of assays have been described for detection of *E. coli* O157:H7 in food *(3)*. However, relatively few of these techniques have been evaluated for detection of low concentrations of pathogens that may have been sublethally injured or stressed by conditions in the food environment and/or during food processing and storage. Ground beef and beef products are generally processed and stored under refrigeration conditions and stored and transported cold or frozen to prevent the growth of spoilage bacteria. A concern is that injured or stressed pathogenic bacteria such as *E. coli* may fail to repair and grow in selective enrichment media because of the presence of selective agents such as bile salts, sodium chloride, or antibiotics or may be overgrown by the background microflora. The medium used for enrichment should facilitate the recovery of the injured target pathogenic bacteria and permit their rapid growth over the competing microbiota.

In other studies, cold-stressed bacteria have had a longer lag phase, thus necessitating longer enrichment times for their detection *(6, 17)*. Uyttendaele et al. *(17)* found that cold stress increased the lag phase of *E. coli* O157:H7 inoculated into ground beef, and these authors recommended

* Author for correspondence. Tel: 215-233-6525; Fax: 215-233-6581; E-mail: pina.fratamico@ars.usda.gov.

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an enrichment time of 24 h to ensure detection of low concentrations (≥1 to 10 CFU/g) of the pathogen. In another report, Uyttendaele et al. *(18)* found that compared with nonstressed samples, meat samples seeded with *Salmonella* and subjected to freezing at −18°C for 7 days produced false-negative results using the iQ-Check PCR assay and the Vidas system. A membrane-immunofluorescent viability staining technique was used for detection of *Salmonella* Enteritidis seeded into ground beef and subjected to freezing, heating, low pH, or high salt conditions *(5)*. Decreases of approximately 1.8 (−20°), 1.3 (2 min, 57°C), 2.4 (4 min, 57°C), 1.5 (2% NaCl), 1.2 (4% NaCl), 1.3 (pH 5.0), and 3.3 (pH 3.5) log CFU/ml were obtained following these treatments when compared with nonstressed bacteria. The staining technique revealed that low pH resulted in the highest concentration of nonviable bacteria, followed by high salt, freezing, and heat. However, the investigators suggested that poor correlation between cell counts obtained from plates and from immunofluorescent viability assays for broth and meat samples (r^2 = 0.28 and r^2 = 0.51, respectively) following freezing was due to sublethal injury of pathogen cells, resulting in damage of the antibody-binding site and reduced membrane integrity. In another study, the lag phase of wild-type and *sigB* (alternative sigma factor σ^B) mutants of *Listeria monocytogenes* inoculated into phosphate-buffered saline and incubated at 4°C for various time periods was extended compared with that of nonstressed bacteria, and this effect was more evident in the *sigB* mutants under certain conditions *(12)*. Although the onset of log growth was delayed in cold-stressed *L. monocytogenes,* the final cell density was not affected.

The objectives of this study were to evaluate the sensitivity of various cultural methods using three different enrichment media and four selective plating media for recovery and detection of cold- and freeze-stressed *E. coli* O157:H7 inoculated at low concentrations into ground beef samples. The sensitivity of the cultural method was compared with that of a commercially available immunoassay and a multiplex PCR assay for detection of the pathogen.

MATERIALS AND METHODS

Bacterial strains and preparation of inocula. *E. coli* O157:H7 strains 380-94 (meat isolate, U.S. Department of Agriculture [USDA], Food Safety and Inspection Service [FSIS], Athens, Ga.), C9490 (clinical isolate, Centers for Disease Control and Prevention, Atlanta, Ga.), and 933 (meat isolate, USDA, FSIS) were inoculated separately into 50 ml of tryptic soy broth (Becton Dickinson, Sparks, Md.) from colonies grown on tryptic soy agar (Becton Dickinson) and were cultured at 37°C for 18 h. A cocktail containing approximately equal numbers of cells from all three strains was diluted in 0.1% peptone water to obtain final concentrations of ca. 10 to 20 and 1 to 2 CFU/ml.

Inoculation of beef and stress conditions. Ground beef (80% fat) was purchased from local retailers, and 25-g samples were placed into 500-ml Erlenmeyer flasks. Each ground beef sample was then inoculated by pipette with 2 ml of the bacterial suspensions to achieve target concentrations of ≤0.5 and ≤2 CFU/g. Each treatment with the different enrichment media consisted of one sample inoculated with the higher cell concentration, two samples inoculated at the lower cell concentration to ensure that at least one of the samples was inoculated with the low concentration of cells, and an uninoculated sample as a negative control. One set of the four treatment samples was subjected to enrichment immediately after inoculation with *E. coli* O157:H7, another set was stored in a freezer at −20°C for 2 weeks, and another set was stored in a refrigerator at 4°C for 3 days prior to enrichment. The samples were held in the flasks during storage.

Enrichment. R&F *E. coli* O157:H7 enrichment broth (RFB; R&F Products, Inc., Downers Grove, Ill.) was prepared by adding 1.0% novobiocin, 1.0% cefsulodin, 0.8% vancomycin, and 0.4% 3-hydroxy-2-methyl-4-pyrone (Sigma, St. Louis, Mo.), as instructed by the manufacturer, to 900 ml of water. The three enrichment media evaluated were RFB, RapidChek *E. coli* O157:H7 enrichment broth (REB; Strategic Diagnostics Inc., Newark, Del.), and modified *E. coli* broth (Becton Dickinson) containing 20 mg/liter novobiocin (mEC+n). Immediately after inoculation (nonstressed) or after storage at 4°C or −20°C (cold and freeze stressed, respectively), 225 ml of an enrichment medium was added to the flasks containing the ground beef samples, which were then incubated at 42°C at 150 rpm. One additional set of enrichments in mEC+n was incubated at 35°C without rotation. Samples for analysis were removed from each flask after 8 and 20 h of enrichment.

Culturing on selective agars. Aliquots of enrichment cultures collected at 8 and 20 h were diluted in 0.1% peptone water and plated onto R&F *E. coli* O157:H7 chromogenic plating medium (R&F Products), cefixime-tellurite sorbitol MacConkey agar (CT-SMAC; CT Supplement, Dynal Biotech, Inc., Lake Success, N.Y.), CHROMagar O157 (Dynal Biotech), and Rainbow agar O157 (Biolog, Inc., Hayward, Calif.). Five milliliters of dimethylformamide, 5 ml of 0.2% novobiocin, and 0.2 ml of 0.1% potassium tellurite were added to 1 liter of R&F *E. coli* O157:H7 chromogenic plating medium; 10 mg of novobiocin and 1.25 mg of potassium tellurite were added to 1 liter of Rainbow agar O157; and 2.5 mg of potassium tellurite were added to 1 liter of CHROMagar. Plates were incubated for 18 h at 37°C, and presumptive *E. coli* O157:H7 colonies (colorless colonies on CT-SMAC, black colonies on Rainbow agar O157, mauve colonies on CHROMagar, and blue-black colonies with black precipitate on R&F *E. coli* O157:H7 chromogenic plating medium) were confirmed to be *E. coli* O157 by O-agglutination (*E. coli* O157 latex test kit, Oxoid, Inc., Nepean, Ontario, Canada). Typical *E. coli* O157:H7 colonies were enumerated, and results were recorded as log CFU per milliliter.

Detection of *E. coli* O157:H7 using the RapidChek *E. coli* O157 lateral flow cassette. Samples from 8- and 20-h enrichment cultures were also tested for the presence of *E. coli* O157:H7 using the RapidChek *E. coli* O157 test kit (Strategic Diagnostics), a lateral flow immunoassay. Several drops of enrichment were added to the well of the cassette using a transfer pipette included with the kit, and after 10 min the results were obtained. Cassettes in which both the control and test bands appeared were recorded as positive.

Detection of *E. coli* O157:H7 using a multiplex PCR assay. The enrichments (1 ml) were subjected to DNA extraction using the PrepMan Ultra reagent (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. The PCR mixture was prepared using the PCR reagent system (Invitrogen, Carlsbad, Calif.) and consisted of 0.25 μM concentrations of each of the primers (AE20-2 and AE22 targeting the *E. coli* O157:H7 *eae* gene and MK1 and MK2 targeting conserved sequences of the stx_1 and stx_2 genes) *(4)*, 3 mM $MgCl_2$, 1.25 U of

TABLE 1. *Comparison of results of detection of nonstressed, cold-stressed, and freeze-stressed* E. coli *O157:H7 in ground beef following enrichment in REB, RFB, and mEC+n using a culture method, the RapidChek* E. coli *O157 lateral flow immunoassay, and a multiplex PCR assay*

Enrichment broth, temp	Detection method	No. of positive samples/total no. of samples analyzed[a]						Total no. (%) of positive samples[b]
		Nonstressed		Cold stressed		Freeze stressed		
		8 h	20 h	8 h	20 h	8 h	20 h	
REB, 42°C	Culture	6/6	6/6	4/5[c]	5/5[c]	5/5[c]	5/5[c]	31/32[c] (97) A
	Immunoassay	6/6	6/6	3/5	3/5	5/5	5/5	28/32 (88) B
	Multiplex PCR	6/6	6/6	5/5	5/5	4/5	5/5	31/32 (97) C
RFB, 42°C	Culture	6/6	6/6	4/6	5/6	6/6	6/6	33/36 (92) A
	Immunoassay	3/6	5/6	6/6	4/6	5/6	6/6	29/36 (81) B
	Multiplex PCR	6/6	5/6	5/6	3/6	3/6	6/6	28/36 (78) D
mEC+n, 42°C	Culture	6/6	6/6	6/6	5/6	6/6	6/6	35/36 (97) A
	Immunoassay	2/6	6/6	2/6	4/6	1/6	6/6	21/36 (58) E
	Multiplex PCR	1/6	4/6	4/6	2/6	2/6	6/6	19/36 (53) F
mEC+n, 35°C	Culture	2/6	2/6	1/6	0/6	1/6	2/6	8/36 (22) G
	Immunoassay	0/6	4/6	0/6	3/6	0/6	4/6	11/36 (31) E
	Multiplex PCR	0/6	4/6	0/6	0/6	0/6	5/6	9/36 (25) H
Total no. (%) of positive samples[d]		44/72 (61) A	60/72 (83) B	40/69 (58) A	39/69 (57) C	38/69 (55) A	62/69 (90) B	283/420 (67)

[a] Each treament consisted of two samples inoculated with *E. coli* at ≤0.5 CFU/g and one inoculated with ≤2 CFU/g. The results of two experiments are shown.

[b] For a given detection method (culture, immunoassay, and PCR), percentages followed by different letters are significantly different ($P < 0.05$).

[c] Two samples, one subjected to cold stress and one to freeze stress, in REB that should have received ≤0.5 CFU/g but were negative were not included in the total number of samples tested.

[d] For a given enrichment time (8 or 20 h), percentages followed by different letters are significantly different ($P < 0.05$).

Taq DNA polymerase, 300 μM concentrations of each of the four dNTPs, and 5.0% dimethyl sulfoxide. Template DNA (2.5 μl) was added, for a total reaction volume of 25 μl. The PCR was performed using a SmartCycler (Cepheid, Sunnyvale, Calif.), and the cycling protocol consisted of 94°C for 2 min and then 35 cycles of 94°C for 20 s, 50°C for 60 s, and 72°C for 60 sec. The PCR products obtained were 224 (stx_2), 227 (stx_1), and 397 (*eae*) bp.

Statistical analyses. The data were analyzed to determine the significance of the effects of the stress treatments, media, inoculum concentrations, and duration of enrichment and of the various types of assays: culture, immunoassay, and multiplex PCR. Logistic regression was used to determine the odds ratio of obtaining a positive result as a function of treatment, medium, inoculum concentration, duration of enrichment, and assay type. Similarity of results between assay types also was investigated by calculating kappa statistics and Cochran's *Q*. The plating data were compared using an analysis of variance, and the means were separated by a Bonferroni least significant difference technique using SAS (SAS/STAT 9.1, SAS Institute, Cary, N.C.). The level of significance for all comparisons was set at $P < 0.05$.

RESULTS AND DISCUSSION

The percentages of samples that were positive by plating were similar for REB, RFB, and mEC+n (42°C) enrichments (97, 92, and 97%, respectively) (Table 1). The percentage of positive samples was significantly lower (22%) for samples subjected to enrichment in mEC+n at 35°C. Overall, higher numbers of *E. coli* O157:H7 cells were obtained using the four agars (Rainbow agar, CT-SMAC, R&F *E. coli* O157:H7 chromogenic plating medium, and CHROMagar O157) with the REB enrichment (4.0 to 7.9 log CFU/ml) than with RFB (1.4 to 7.4 log CFU/ml), mEC+n incubated at 42°C (1.7 to 6.7 log CFU/ml), and mEC+n incubated at 35°C (1.3 to 3.3 log CFU/ml) (Table 2). Generally, the concentrations were higher after 20 h of enrichment than after 8 h; and this difference was significant for freeze-stressed cells, except for samples enriched in mEC+n incubated at 35°C. The concentrations recovered from samples held at 4°C for 3 days were much lower than those from samples with nonstressed and freeze-stressed bacteria. In all samples enriched in mEC+n and incubated at 35°C, there were significantly lower concentrations recovered on all of the agars compared with enrichments in mEC+n at 42°C, REB, and RFB. Among the four different agars tested, there was no notable difference in the number of *E. coli* O157:H7 colonies enumerated from enriched cultures.

Grant *(8)* compared a new method involving an acidic enrichment procedure with five standard methods for detection of *E. coli* O157:H7 inoculated at 1 CFU/g in different types of food. Recovery of the organism using the acid enrichment procedure was significantly greater than that using the USDA method *(16)* based on plating onto Rainbow agar containing potassium tellurite and novobiocin and on a PCR assay. The USDA method involves the use of mEC+n for enrichment and Rainbow agar O157 for plating the enriched cultures; therefore, selection of the appropriate enrichment medium is important. In a comparison of Rainbow agar O157, BCM O157:H7, Fluorocult HC, and sorbitol MacConkey agar for isolation of *E. coli* O157:

TABLE 2. *Average concentrations of nonstressed, cold-stressed, and freeze-stressed* E. coli *O157:H7 after plating 8- and 20-h REB, RFB, and mEC+n enrichments on four plating media, and results of the RapidChek* E. coli *O157 lateral flow immunoassay and multiplex PCR assay*[a]

Enrichment broth, temp, and initial inoculum concn	Nonstressed						Cold stressed						Freeze stressed					
	8 h			20 h			8 h			20 h			8 h			20 h		
	Concn (CFU/ml)	Immuno-assay[b]	PCR[b]	Concn (CFU/ml)	Immuno-assay	PCR	Concn (CFU/ml)	Immuno-assay	PCR	Concn (CFU/ml)	Immuno-assay	PCR	Concn (CFU/ml)	Immuno-assay	PCR	Concn (CFU/ml)	Immuno-assay	PCR
REB, 42°C																		
≤0.5 CFU/g	5.01 ± 0.55	4/4	4/4	5.71 ± 0.77	4/4	3/4	4.11 ± 0.87	1/3[c]	3/3	4.69 ± 0.71	1/3[c]	3/3	3.99 ± 1.64	3/3[c]	2/3	5.64 ± 2.68	3/3[c]	3/3
≤2 CFU/g	6.07 ± 0.70	2/2	2/2	6.52 ± 1.57	2/2	2/2	5.03 ± 0.55	2/2	2/2	5.54 ± 0.61	2/2	2/2	5.72 ± 0.34	2/2	2/2	7.85 ± 1.10	2/2	2/2
RFB, 42°C																		
≤0.5 CFU/g	2.27 ± 0.43	1/4	4/4	3.78 ± 1.37	3/4	3/4	1.42 ± 0.28	2/4	3/4	1.82 ± 0.52	0/4	2/4	2.12 ± 0.43	3/4	2/4	6.83 ± 0.66	4/4	4/4
≤2 CFU/g	3.66 ± 1.10	2/2	2/2	3.73 ± 1.42	2/2	2/2	2.40 ± 0.21	2/2	2/2	2.18 ± 0.97	2/2	1/2	2.82 ± 0.38	2/2	1/2	7.37 ± 0.84	2/2	2/2
mEC+n, 42°C																		
≤0.5 CFU/g	3.18 ± 0.80	1/4	1/4	4.81 ± 0.86	4/4	2/4	1.69 ± 0.58	1/4	2/4	2.52 ± 1.02	1/4	1/4	2.62 ± 1.33	0/4	1/4	5.58 ± 0.63	4/4	4/4
≤2 CFU/g	3.53 ± 0.58	1/2	0/2	5.25 ± 1.10	2/2	2/2	2.71 ± 1.17	0/2	2/2	3.85 ± 1.28	1/2	1/2	3.34 ± 0.56	1/2	1/2	6.66 ± 0.81	2/2	2/2
mEC+n, 35°C																		
≤0.5 CFU/g	1.82 ± 0.51	0/4	0/4	3.31 ± 0.79	2/4	2/4	≤1.32	0/4	0/4	1.82 ± 0.52	0/4	0/4	≤1.32	0/4	0/4	1.50 ± 0.50	2/4	3/4
≤2 CFU/g	1.90 ± 0.63	0/2	0/2	3.32 ± 1.07	2/2	2/2	1.67 ± 0.81	0/2	0/2	1.82 ± 0.53	1/2	0/2	1.66 ± 0.51	0/2	0/2	≤1.32	2/2	2/2

[a] The four plating media were CT-SMAC, Rainbow agar O157, R&F *E. coli* O157:H7 chromogenic plating medium, and CHROMagar O157. Each treatment consisted of two samples inoculated with ≤0.5 CFU/g and one inoculated with ≤2 CFU/g. The results of two experiments are shown.

[b] Results are given as the no. of positive samples/no. of samples tested.

[c] Two samples, one subjected to cold stress and one to freeze stress, in REB that should have received ≤0.5 CFU/g but were negative were not included in the total number of samples tested.

H7 from food known to be negative for the pathogen, false-positive result rates were 2.1, 3.3, 6.2, and 57.3%, respectively *(11)*. The presence of sorbitol-negative colonies formed by bacteria other than *E. coli* O157:H7 resulted in the high percentage of false-positive results obtained, particularly on sorbitol MacConkey agar. The addition of cefixime and potassium tellurite to SMAC increases the sensitivity and specificity for detection of *E. coli* O157:H7, as demonstrated by Onoue et al. *(13)*. These investigators found that other selective agars tested, including CHROMagar O157, BCM O157, and CT-SMAC, in combination with enrichment in mEC+n at 42°C were more sensitive than SMAC for detection of *E. coli* O157:H7 inoculated at a concentration of ca. 24 CFU per 25 g of ground beef.

E. coli O157:H7 was detected by the RapidChek *E. coli* O157 immunoassay in all samples enriched in REB except for two samples with cold-stressed bacteria inoculated at ca. ≤0.5 CFU/g after both 8 and 20 h of enrichment (Tables 1 and 2). The percentages of positive results with the immunoassay using the REB and RFB enrichments (88 and 81%, respectively) were significantly different than the results obtained using mEC+n incubated at 42°C (58%) and mEC+n incubated at 35°C (31%). Only 31% of the samples were positive using the mEC+n enrichments incubated at 35°C, probably because of the presence of low numbers of *E. coli* O157:H7 cells (Tables 1 and 2). Onoue et al. *(13)* found that the sensitivity of immunoassays for detection of *E. coli* O157:H7 on radish sprouts and in ground beef samples was similar to that of plating in conjunction with immunomagnetic separation, although the specificity of the immunoassays was lower than that for plating. In a previous study, we found poor agreement between results obtained with an immunoassay and those obtained with plating or PCR methods for detection of *Salmonella* in ground beef, chicken, and turkey samples, probably because of the poor sensitivity and specificity of the immunoassay *(7)*. In the current study, statistical analyses revealed that the odds of obtaining a positive result (detecting *E. coli* O157:H7 at an initial inoculum concentration of ≤0.5 CFU/g) were ca. 2.5 times more likely with plating than with the immunoassay or PCR assay. The higher sensitivity of the plating method may have been associated with the fact that the samples were plated onto four agars instead of one, and results were recorded as positive when *E. coli* O157:H7 colonies were detected on at least one of the four agars. Arthur and coworkers *(1)* compared results of three PCR-based methods with those of two culture methods for detection of *E. coli* O157:H7 in ground beef. A higher number of positive samples was obtained with the culture-based procedures than with the PCR assays; however, the culture-based methods required at least 9 h longer to obtain results than did the PCR methods. In the current study, none of the uninoculated control samples were positive with the immunoassay; thus although we did not test a very large number of samples, the specificity of the RapidChek *E. coli* O157 lateral flow immunoassay was satisfactory. However, an investigation comparing the performance of the RapidChek immunoassay with that of other similar commercially available lateral flow immunoassays should be conducted.

The multiplex PCR assay evaluated for detection of *E. coli* O157:H7 from the enrichment broths targeted conserved sequences of the *E. coli* O157:H7 stx_1 and stx_2 genes and the *eae* gene. Similar to results obtained with the immunoassay, the percentages of positive samples obtained using the multiplex PCR assay were 97% with REB, 78% with RFB, 53% with mEC+n at 42°C, and 25% with mEC+n at 35°C (Table 1). All percentages were significantly different from each other based on logistic regression analysis. In a study comparing plating, PCR, TaqMan-based real-time PCR, and an immunoassay for detection of *Salmonella* in naturally contaminated ground chicken, turkey, and beef, the PCR assays were more sensitive than plating for the ground chicken and turkey samples, and the real-time PCR assay was more sensitive than conventional PCR with detection of product by agarose gel electrophoresis *(7)*. In the current study, sensitivity may have been higher if a real-time PCR assay rather than a conventional PCR had been used, or possibly further optimization of the PCR assay was needed.

Logistic regression analyses of the data indicated that there were significant effects of stress treatment, enrichment medium and incubation time, inoculum concentration, and assay type. In particular, a positive result was 1.1 times more likely to be obtained after 20 h of enrichment than after 8 h and 25 times more likely with RFB and REB than with mEC+n at 35°C. The odds of obtaining a positive result were 10 times more likely with mEC+n at 42°C than with mEC+n at 35°C, 3.7 times more likely with the higher inoculum concentration (≤2.0 CFU/g) than the lower concentration (≤0.5 CFU/g), 2.5 to 3 times more likely for freeze-stressed or nonstressed bacteria than for cold-stressed bacteria, and 2.5 to 2.7 times more likely by plating than by immunoassay or PCR assay. In 28 of 144 samples, *E. coli* O157:H7 was not detected on any of the four agars, either because the cell numbers were too low after enrichment or colonies of background flora obscured those of the *E. coli* O157:H7. Therefore, if only one selective agar had been used rather than four, the sensitivity of the plating method may have been lower. We obtained aerobic plate counts for the enriched cultures after 8 and 20 h of incubation. In the samples obtained after 20 h of enrichment, the concentrations ranged from 8 to 9 log CFU/ml. After 8 h of enrichment, the concentration was 6.7 to 8.7 log CFU/ml in REB, 3.6 to 6.3 log CFU/ml in RFB, 4.6 to 6.9 log CFU/ml in mEC+n at 42°C, and 3.3 to 6.8 log CFU/ml in mEC+n at 35°C (data not shown). Thus, *E. coli* O157:H7 may have been detected by the immunoassay and by the PCR assay after 20 h of enrichment in mEC+n at 35°C but not by plating because of the high number of colonies of background microflora that obscured the *E. coli* O157:H7 colonies (Table 2).

Physiological changes in bacteria in response to exposure to low temperatures include changes in membrane fatty acid composition resulting in decreased membrane fluidity, stabilization of nucleic acid secondary structures resulting in decreased transcription and translation, synthesis

of cold stress proteins, importation of compatible solutes, and/or alterations in ribosomes *(2, 14)*. Freezing and thawing cause cell membrane and DNA damage. Thus, the cold or freeze stress treatments could have inactivated or injured the *E. coli* O157:H7 in the ground beef resulting in lower numbers of positive samples, in particular for samples inoculated at ≤0.5 CFU/g. The lower number of positive samples after 8 h of enrichment compared with 20 h may have been due to an increased bacterial lag time as a result of stress *(17)*. However, the only significant effects due to enrichment time (8 versus 20 h) before plating were observed for the freeze-stressed samples grown in the REB, RFB, and mEC+n (42°C) broths (Table 2). Overall, there were no notable differences in the number of positive samples for all of the methods after 20 h of enrichment compared with 8 h, except for samples (nonstressed and cold and freeze stressed) subjected to enrichment in mEC+n at 35°C and tested using the PCR assay and the immunoassay (Table 1). The percentage of positive samples by all methods used (58%) was the same after 8 and 20 h of enrichment in ground beef samples containing cold-stressed *E. coli* O157:H7 (Table 1). In cold-stressed samples, the concentrations obtained by plating after 8 and 20 h of enrichment were higher with REB than with the other media (Table 2).

Lionberg et al. *(10)* found that the sensitivity for detecting freeze-injured *E. coli* O157:H7 by the PCR assay and by plating was higher with RFB than with buffered peptone water containing vancomycin, cefsulodin, and cefixime (BPW-VCC) or mEC+n. After 6 h of enrichment, 47.6, 19.1, and 9.5% of 21 samples inoculated with freeze-injured *E. coli* O157:H7 at ca. 1.5 CFU per 25 g were positive by the PCR using RFB, BPW-VCC, and mEC+n, respectively, and after 8 h of enrichment, the percentages increased to 95.2, 61.9, and 71.4%, respectively. Thus, RFB was superior to BPW-VCC and mEC+n. Similarly, in the current study, the total number of positive results obtained with the immunoassay and the PCR assay was higher when RFB rather than mEC+n was used for enrichment (Table 1). Uyttendaele et al. *(17)* observed that a 3-h increase in enrichment time was needed for PCR detection of *E. coli* O157:H7 inoculated in ground beef samples that were stored at 4°C for 4 days compared with samples that contained nonstressed bacteria. Similar results were obtained for ground beef stored at −20°C for up to 14 days. The authors indicated that the increase in enrichment time was needed due to an increase in the lag phase caused by cold stress.

In the current study, the number of positive ground beef samples was lower for samples containing cold-stressed bacteria than for those containing nonstressed and freeze-stressed *E. coli* O157:H7, even after 20 h of enrichment. The reason we obtained higher sensitivities by plating, the immunoassay, and the PCR assay for samples with nonstressed and freeze-stressed *E. coli* O157:H7 is unclear. During exposure to 4°C for 3 days, psychrotrophic bacteria may have grown in the meat, and a change in the microbial flora of the ground beef may have had an impact on subsequent recovery of *E. coli* O157:H7. Using temporal temperature gel electrophoresis and denaturing gradient gel electrophoresis, Lafarge et al. *(9)* found that after storage of raw cow milk for 24 h at 4°C, the relative proportions of bacteria were altered. In several cases, new bands appeared on the gels after refrigeration.

In the current study, *E. coli* O157:H7 was not detectable by any of the methods used in one sample enriched in REB and subjected to cold stress and in one sample enriched in REB and subjected to freeze stress; each had been inoculated with ≤0.5 CFU/g. There are two possibilities: (i) the sample did not receive any *E. coli* O157:H7 when inoculated because of the low concentration in the inoculum or (ii) the bacteria died during the cold- or freeze-stress treatments. It seems more likely that the meat did not receive any cells when inoculated, because this completely negative result was obtained on only these two occasions, and *E. coli* O157:H7 was detected in the other sample inoculated at the same concentration in each of the two treatments. Therefore, the two samples that should have received ca. 0.5 CFU/g but tested negative after enrichment were not included in the total number of samples tested (Tables 1 and 2).

In conclusion, higher sensitivity for detection of *E. coli* O157:H7 with the immunoassay and the PCR assay was obtained for ground beef samples subjected to enrichment in REB and RFB compared with mEC+n incubated at 42°C and particularly mEC+n incubated at 35°C. Overall, the highest percentages of positive samples by plating, the immunoassay, and the multiplex PCR assay were obtained with samples enriched in REB, and the cell numbers were generally higher following plating of REB enrichments for nonstressed, cold-stressed, and freeze-stressed *E. coli* O157:H7 in ground beef compared with RFB, mEC+n (42°C), and mEC+n (35°C) enrichments. Thus, our results indicate that REB is superior to the other media for enrichment of cold-stressed and freeze-stressed *E. coli* O157:H7 present in low concentrations in ground beef. REB likely permits rapid repair of bacterial injury that may have occurred during exposure to cold or freezing temperatures, although both REB and RFB were superior to enrichment in mEC+n, in particular, when the mEC+n enrichments were incubated at 35°C. Further studies are needed to examine the effects of additional stressors, such as acid and heat, on recovery of *E. coli* O157:H7 enriched in REB.

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